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TRIBAL PHYLOGENY OF THE *Randall J. Bayer*<sup>2,3</sup> and *Julian R. Starr*<sup>4</sup>  
ASTERACEAE BASED ON  
TWO NON-CODING  
CHLOROPLAST SEQUENCES,  
THE *trnL* INTRON AND *trnL*/  
*trnF* INTERGENIC SPACER<sup>1</sup>

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ABSTRACT

Asteraceae are the largest family of dicotyledonous plants and have long been known for their taxonomic complexity. The ubiquitous parallelisms in morphology within the family have made phylogenetic reconstruction and tribal circumscription an area of long debate. In this study we explored the utility of using two relatively short non-coding chloroplast DNA sequences, the *trnL* intron and *trnL/trnF* intergenic spacer, to resolve phylogenetic relationships among the tribes. The results of the phylogenetic analysis produced trees that are topologically congruent with prior phylogenetic hypotheses based on both morphological and molecular data sets. The Asteroideae are a monophyletic group, but the Cichorioideae are paraphyletic. The primary clades of the Cichorioideae are the Mutisaceae–Cardueae, Liabeae–Vernonieae, and of the Asteroideae, the Inuleae–Plucheeae, Astereae–Anthemideae, Senecioneae–Gnaphalieae, and the helianthoid clade (Helenieae, Heliantheae s. str., and Eupatorieae). The Inuleae–Plucheeae clade is sister to the remainder of the Asteroideae, and the paraphyly of the Inuleae s.l. (Gnaphalieae, Inuleae s. str., and Plucheeae) is firmly supported by our analysis. Our study illustrates the utility of the *trnL* intron and *trnL/F* intergenic spacer for resolving relationships among tribes of the Asteraceae. Using approximately 874 bp, we were able to produce a phylogeny of comparable resolution to phylogenies based on well-known coding regions such as *rbcl* and *ndhF*. For phylogenetic inference at the family level the *trnL* intron and *trnL/F* spacer provide similar levels of resolution to longer coding sequences (e.g., *rbcl*, *ndhF*), while having the advantage of being much easier to amplify and sequence due to their short lengths and universal primers. The numerous insertions and deletions commonly found in this region are easily aligned and are phylogenetically informative, thus adding considerably to the information content per base pair sequenced.

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Asteraceae are the largest family of dicotyledonous plants (ca. 23,000 spp.) and have long been recognized for their taxonomic complexity. Ubiquitous parallelisms in morphology within the family have made it difficult to find conservative (non-homoplasious) characters that can be used reliably in phylogenetic reconstruction (Carlquist, 1976). Cassini (1826) was the first to divide the Asteraceae into tribes (19 tribes), and the first to suggest their natural relationships. Significant early contributions were also made by Bentham (1873), who reduced the number of tribes to 13, and Cronquist (1955), who placed Heliantheae at the base of his 12 recircumscribed tribes. Hoffmann (1894) rec-

ognized two distinct lineages within the Asteraceae: the Liguliflorae, in which he placed the single tribe Lactuceae; and the Tubuliflorae (= Asteroideae of modern authors), in which he placed all the remaining tribes. Subsequent authors have continued to recognize two lineages within the family, but their circumscriptions have differed dramatically. Among these major revisions, Carlquist (1976) was perhaps the first to recognize an expanded Cichorioideae (= Liguliflorae) by placing 6 tribes within each of his subfamilies Cichorioideae and Asteroideae. Beginning in the late 1980s, the discovery and subsequent analysis of a phylogenetically informative inversion in the cpDNA of Asteraceae

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(Jansen & Palmer, 1987), in addition to the morphological work by Bremer (1987) and others, demonstrated that the former Barnadesiinae (in Mutisieae) was monophyletic. This work also indicated that this subtribe was the basal group in the Asteraceae and worthy of being recognized as the archaic subfamily, the Barnadesioideae. As a result of these and other morphological and molecular studies (Bremer, 1987; Michaels et al., 1993; Gustafsson & Bremer, 1995; Kim & Jansen, 1995), it is becoming clear that the Asteraceae arose in South America (Bremer, 1992) and are probably sister to the South American endemic family Calyceraceae.

Phylogenetic relationships within the Asteraceae have long been an area of debate, beginning with Cassini (1826) and continuing to the present day. Although much has been accomplished over the past 15 years to resolve phylogenetic relationships among the tribes, the taxonomic limits and relationships of many tribes are still unclear. In particular, the question of the monophyly of the Cichorioideae and the "old" Inuleae are important relationships that have not been resolved. In addition, the tribal circumscriptions of tribes such as the Helenieae and Eupatorieae are still very much in doubt.

Since the advent of molecular systematics, protein-encoding gene sequences have been very useful for resolving higher-order questions (e.g., Chase et al., 1993). However, in groups such as the Asteraceae that have undergone a rapid radiation (Carlquist, 1976), coding regions may not always provide sufficient information to resolve relationships. In this study we explored the utility of using two relatively short, non-coding chloroplast DNA sequences, the *trnL* intron and *trnL*/*trnF* intergenic spacer, to resolve phylogenetic relationships among tribes of the Asteraceae. The availability of *rbcl* (Kim et al., 1992) and *ndhF* (Kim & Jansen, 1995) derived phylogenetic trees allows for a direct comparison of the phylogenetic utility of the *trnL* intron and *trnL*/*trnF* intergenic spacer relative to these widely used sequences.

## MATERIALS AND METHODS

### OUTGROUP SELECTION

Outgroup taxa were selected on the basis of the *ndhF* analysis of Kim and Jansen (1995), the restriction fragment length polymorphism (RFLP) studies by Jansen and Palmer (1987, 1988), the *rbcl* analysis by Kim et al. (1992), and the morphological works of Bremer (1987, 1994). Although attempts were made to use groups from outside the Asteraceae to polarize trees, alignments were am-

biguous and could not be used for phylogenetic reconstruction. Two members of the Barnadesioideae (i.e., *Chuquiraga* and *Doniophyton*) were thus chosen as a functional outgroup (Watrous & Wheeler, 1981). The basal position of this subfamily is confirmed in all the above-mentioned studies, and its use as an outgroup for the remainder of the Asteraceae is not without precedent (Jansen et al., 1990, 1991; Keeley & Jansen, 1991).

### INGROUP SAMPLING

Tribal circumscriptions and nomenclature are based on the treatment of the Asteraceae by Bremer (1994). One or two members from each of the recognized tribes were sequenced. For the 26 taxa used in this study, all sequences were generated by us (Table 1) from fresh leaf material, except for representatives of *Artemisia*, *Chuquiraga*, *Doniophyton*, *Liabum*, and *Osteospermum*, which were obtained from dried material. Material was collected in the field for some genera, whereas other samples were obtained from commercial sources; herbarium vouchers are cited in Table 1.

### DNA ISOLATION, AMPLIFICATION, AND SEQUENCING

Total DNA was isolated as outlined in Bayer et al. (1996). The *trnL*/*F* region was amplified via the polymerase chain reaction (PCR) using *Taq* DNA polymerase on a GeneE<sup>®</sup> thermal cycler (Techne Incorporated, Princeton, NJ). The PCR reaction mixture consisted of 5 µl of 20× reaction buffer, 6 µl of 25 mM magnesium chloride solution, 16 µl of a 1.25 mM dNTP solution in equimolar ratio, 25 pmol of each primer, 10–50 ng of template DNA, and 1.0 unit of polymerase in a total volume of 100 µl. The PCR samples were heated to 94°C for three minutes prior to the addition of DNA polymerase to denature unwanted proteases and nucleases. The double-stranded PCR products were produced via 30 cycles of denaturation (94°C for 1.0 min), primer annealing (48°C for 1 min), and extension (72°C for 2 min). A 7-min final extension cycle at 72°C followed the 30th cycle to ensure the completion of all novel stands.

The *trnL* intron and *trnL*/*trnF* spacer, hereafter referred to as *trnL5'/F* (Fig. 1), was amplified as a single piece using primers "c" and "f" of Taberlet et al. (1991). Primers "a" and "b" (Fig. 1) were used to estimate the approximate size of the *trnT*/*L* intergenic spacer in the Asteraceae, but these were not sequenced. Double-stranded PCR products were cleaned by differential filtration using Millipore Ultra-free<sup>®</sup>-MC tubes (30,000 NMWL filters) prior to sequencing.

The double-stranded PCR products were then

Table 1. Collections of Asteraceae used in the *trnL/trnT* sequencing study. Presented are species, origin (location of voucher), and accession numbers. All voucher numbers beginning with two letters (signifying a state, province, or from cultivation (GH)) followed by 5 digits are collections of Bayer or Bayer et al. GenBank accession numbers for the sequences (intron, spacer) are given.

| Species   | Accession numbers and<br>(voucher location) | Source                       | GenBank<br>(intron, spacer) |
|---|---|------------------------------|-----------------------------|
| 1) <i>Ageratum houstonianum</i> Mill.                         | GH-95011 (CANB)                             | Commercially grown plants    | U82012, U82013              |
| 2) <i>Antennaria luzuloides</i> Torr. &<br>A. Gray            | OR-91002 (ALTA)                             | U.S.A.: Oregon               | U82014, U82015              |
| 3) <i>Artemisia tridentata</i> Nutt.                          | CO-90072 (ALTA)                             | U.S.A.: Colorado             | U82016, U82017              |
| 4) <i>Aster novae-angliae</i> L.                              | AB-95003 (CANB)                             | Commercially grown plants    | U82018, U82019              |
| 5) <i>Calendula officinalis</i> L.                            | GH-95009 (CANB)                             | Commercially grown plants    | U82020, U82021              |
| 6) <i>Chuiriraga aurea</i> Skottsbl.                          | Stuessy et al. 12911 (OS)                   | Argentina                    | U82022, U82023              |
| 7) <i>Cirsium subniveum</i> Rydb.                             | WY-90044A (CANB)                            | U.S.A.: Wyoming              | U82024, U82025              |
| 8) <i>Crepis tectorum</i> L.                                  | AB-95002 (CANB)                             | Canada: Alberta              | U82026, U82027              |
| 9) <i>Doniophyton anomalum</i> (D.<br>Don) Wedd.              | Stuessy et al. 12857 (OS)                   | Argentina                    | U82028, U82029              |
| 10) <i>Echinops exaltatus</i> Schrad.                         | AB-95005 (CANB)                             | Commercially grown plants    | U82030, U82031              |
| 11) <i>Gaillardia aristata</i> Pursh                          | GH-95006 (CANB)                             | Commercially grown plants    | U82032, U82033              |
| 12) <i>Gazania rigens</i> R. Br.                              | GH-95012 (CANB)                             | Commercially grown plants    | U82034, U82035              |
| 13) <i>Gerbera jamesonii</i> Bolus ex<br>Hook.                | GH-95004 (CANB)                             | Commercially grown plants    | U82036, U82037              |
| 14) <i>Helianthus annuus</i> L.                               | GH-95007 (CANB)                             | Commercially grown plants    | U82038, U82039              |
| 15) <i>Inula helenium</i> L.                                  | GH-95013 (CANB)                             | Commercially grown plants    | U82040, U82041              |
| 16) <i>Lactuca sativa</i> L.                                  | AB-95007 (CANB)                             | Commercially grown plants    | U82042, U82043              |
| 17) <i>Liabum solidagineum</i> (Kunth)<br>Less.               | Dillon & Sánchez 6253 (F)                   | Peru: Prov. Huancabamba      | U82044, U82045              |
| 18) <i>Matricaria matricarioides</i><br>(Less.) Port.         | AB-95005 (CANB)                             | Canada: Alberta              | U82046, U82047              |
| 19) <i>Osteospermum clandestinum</i><br>(Less.) Norl.         | WA-94070 (CANB)                             | Australia: Western Australia | U82048, U82049              |
| 20) <i>Petasites frigidus</i> (L.) Fr.                        | Starr 96001 (WIN)                           | Canada: Manitoba             | U82050, U82051              |
| 21) <i>Senecio vulgaris</i> L.                                | AB-95006 (CANB)                             | Canada: Alberta              | U82052, U82053              |
| 22) <i>Stokesia laevis</i> Greene                             | GH-95014 (CANB)                             | Commercially grown plants    | U82054, U82055              |
| 23) <i>Streptoglossa cylindriceps</i> (J.<br>M. Black) Dunlop | WA-94049 (ALTA)                             | Australia: Western Australia | U82056, U82057              |
| 24) <i>Stuartina muelleri</i> Sond.                           | Burrous s.n. (CANB)                         | Australia: New South Wales   | U82058, U82059              |
| 25) <i>Tagetes patula</i> L.                                  | Bayer s.n. (CANB)                           | Commercially grown plants    | U82060, U82061              |
| 26) <i>Townsendia exscapa</i> (Richard-<br>son) Porter        | CO-93037 (CANB)                             | U.S.A.: Colorado             | U82062, U82063              |

used as templates in cycle sequencing reactions, which employed three primers (Taberlet et al., 1991) to sequence the two regions, including the terminal primers “c” and “f” and an internal primer “d” (Fig. 1). Sequencing primers were 5’ end-labeled in a preliminary reaction involving T4 polynucleotide kinase and [ $\gamma$ - $^{32}$ P] - dATP (Amersham). The double-stranded DNAs were then cycle-sequenced using the dideoxy chain termination method (Sanger et al., 1977) with use of Promega’s *fmo*®\*1 Sequencing System (Promega Corporation, Madison, Wisconsin). An annealing temperature of 57°C was used for primer “f,” while temperatures ranging from 60 to 62°C were employed for primers “c” and “d.” The cy-

cle-sequencing protocol followed the manufacturer’s instructions. Termination products were separated in 6.0% polyacrylamide gels (0.4 mm thickness; 1× TBE buffer); the gels were fixed in 10% acetic acid for 20 minutes, washed in distilled water, and allowed to air-dry. They were then used to expose Kodak BIOMAX®-MR film for 8–48 hr depending on the intensity of the radioactive signal from the gel.

SEQUENCE ANALYSIS AND PHYLOGENETIC  
RECONSTRUCTION

Sequences were aligned initially using CLUSTAL V (Higgins et al., 1992), then adjusted man-

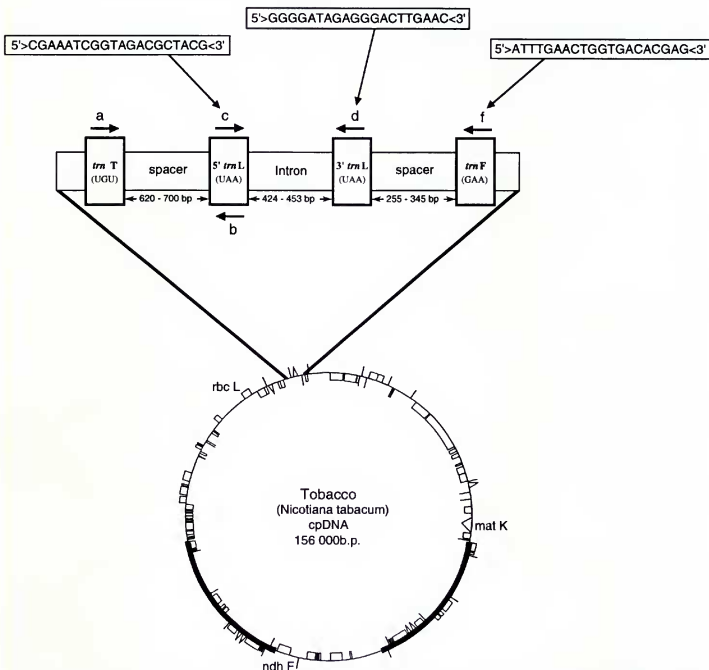


Figure 1. Structure of chloroplast DNA in *Nicotiana tabacum* L. (Solanaceae). Presented are positions of the *trnT* exon (UGU), the *trnL*/T intergenic spacer, the *trnL* intron, the *trnL* 3' and 5' exons (UAA), the *trnL*/F intergenic spacer, and the *trnF* exon (GAA), relative to the commonly sequenced genes *rbcL*, *matK*, and *ndhF*, the large and small single-copy regions, and the inverted repeats (two bold semicircular regions). Relative positions of the Taberlet et al. (1991) primers (c, d, and f) used in PCR and sequencing are indicated, along with their base sequences.

ually (Swofford & Olsen, 1990) to minimize gap number using SeqApp vers. 1.8A (Gilbert, 1992). Several divergence weights [20%, 40%, 60% (the default), and 80%] were explored during sequence alignment (Delay Divergence Option of Clustal V), including several combinations of the gap-opening penalty (GOP) and gap-extension penalty (GEP) options of CLUSAL V (Higgins et al., 1992). GOPs of 10 (the default) and 100 were explored in all permutations with GEPs of 5 (the default) and 10. The different permutations resulted in very similar alignments, and one was chosen as a starting point to continue with manual adjustment of the alignment. The alignment of the sequences necessitated inference of many insertions and deletions (Table 2).

Small portions of the *trnL* and *trnF* genes were also sequenced along with the intron and spacer sequences. No variation was observed among the taxa for any of these gene regions with the exception of a single point mutation (C→T) at the 3<sup>rd</sup> position of the 5' segment of *trnF*. *Artemisia*, *Aster*, *Lactuca*, *Matricaria*, *Petasites*, *Senecio*, and *Townsendia* have "T" at this position, whereas all other taxa have a "C." This character was included in all analyses.

The proportion of nucleotide differences between taxa was calculated using the "Show Distance Matrix" option of PAUP. A total of 101 phylogenetically informative base pairs and 32 indels from the *trnL*5'/F region was available for use in the analysis

Table 2. Insertions and deletions in the chloroplast *trnL* intron and the *trnL*/F intergenic spacer in the Asteraceae. Presented are type and size of the indel, start point of the indel based on the first bp of the intron sequence (*trnL* intron is 1–533; *trnL*/F spacer is 534–913), and the species in which the mutation occurs (numbers of the species refer to those given in Table 1). Also given are the repeat sequences for those insertions that are repeats of adjacent sequences, as well as the locus of the start point from which the repeat is derived. \* = potentially phylogenetically informative indels.

| Indel number | Type of mutation | Size in (bp) | Direct repeat sequence | Fragment from base | Repeated from base | Mutated species       |
|--------------|------------------|--------------|------------------------|--------------------|--------------------|-----------------------|
| 1*           | del              | 1            |                        | 144                |                    | 2, 24, 26             |
| 2*           | del              | 1            |                        | 205                |                    | 4, 26                 |
| 3*           | del              | 1            |                        | 264                |                    | 5, 19                 |
| 4*           | del              | 1            |                        | 273                |                    | 4, 6, 7               |
| 5*           | del              | 1            |                        | 293                |                    | 1, 11, 14, 25         |
| 6            | del              | 1            |                        | 316                |                    | 9                     |
| 7*           | del              | 1            |                        | 388                |                    | 3, 18                 |
| 8*           | del              | 1            |                        | 402                |                    | 1, 11, 14, 15, 23, 25 |
| 9            | del              | 1            |                        | 591                |                    | 1                     |
| 10*          | del              | 1            |                        | 755                |                    | 2, 24                 |
| 11*          | del              | 1            |                        | 844                |                    | 1, 11, 14, 25         |
| 12           | del              | 1            |                        | 887                |                    | 15                    |
| 13           | del              | 2            |                        | 303                |                    | 9                     |
| 14           | del              | 2            |                        | 309                |                    | 9                     |
| 15*          | del              | 2            |                        | 606                |                    | 8, 17, 22             |
| 16           | del              | 2            |                        | 670                |                    | 2                     |
| 17*          | del              | 2            |                        | 672                |                    | 3, 18, 20, 21, 24, 26 |
| 18           | del              | 2            |                        | 679                |                    | 20                    |
| 19           | del              | 2            |                        | 763                |                    | 20                    |
| 20           | del              | 3            |                        | 602                |                    | 17                    |
| 21           | del              | 4            |                        | 189                |                    | 25                    |
| 22*          | del              | 4            |                        | 283                |                    | 1, 11, 14, 25         |
| 23           | del              | 4            |                        | 295                |                    | 19                    |
| 24           | del              | 4            |                        | 332                |                    | 26                    |
| 25           | del              | 4            |                        | 592                |                    | 6                     |
| 26*          | del              | 4            |                        | 621                |                    | 4, 26                 |
| 27           | del              | 4            |                        | 660                |                    | 20                    |
| 28           | del              | 5            |                        | 432                |                    | 21                    |
| 29           | del              | 5            |                        | 598                |                    | 9                     |
| 30           | del              | 5            |                        | 743                |                    | 22                    |
| 31           | del              | 5            |                        | 875                |                    | 13                    |
| 32           | del              | 6            |                        | 666                |                    | 13                    |
| 33*          | del              | 7            |                        | 635                |                    | 9, 16                 |
| 34           | del              | 8            |                        | 115                |                    | 17                    |
| 35           | del              | 8            |                        | 598                |                    | 19                    |
| 36*          | del              | 8            |                        | 682                |                    | 15, 17, 22            |
| 37           | del              | 8            |                        | 806                |                    | 17, 22                |
| 38           | del              | 9            |                        | 420                |                    | 13                    |
| 39           | del              | 9            |                        | 617                |                    | 19                    |
| 40*          | del              | 9            |                        | 654                |                    | 1, 11, 14             |
| 41*          | del              | 9            |                        | 747                |                    | 20, 21                |
| 42           | del              | 9            |                        | 798                |                    | 5                     |
| 43*          | del              | 10           |                        | 115                |                    | 5, 19                 |
| 44           | del              | 10           |                        | 602                |                    | 6                     |
| 45           | del              | 10           |                        | 690                |                    | 9                     |
| 46*          | del              | 10           |                        | 754                |                    | 15, 23                |
| 47*          | del              | 10           |                        | 835                |                    | 5, 19                 |
| 48*          | del              | 11           |                        | 123                |                    | 6, 7                  |
| 49*          | del              | 11           |                        | 761                |                    | 5, 6, 7, 19           |
| 50*          | del              | 15           |                        | 777                |                    | 3, 18                 |

Table 2. Continued.

| Indel number | Type of mutation | Size in (bp) | Direct repeat sequence         | Fragment from base | Repeated from base | Mutated species                        |
|--------------|------------------|--------------|--------------------------------|--------------------|--------------------|--|
| 51*          | del              | 18           |                                | 295                |                    | 3, 18                                  |
| 52           | del              | 19           |                                | 652                |                    | 16                                     |
| 53           | del              | 82           |                                | 730                |                    | 25                                     |
| 54           | ins              | 1            |                                | 141                |                    | 13                                     |
| 55           | ins              | 1            |                                | 143                |                    | 3                                      |
| 56*          | ins              | 1            |                                | 613                |                    | 3, 5, 9, 18, 19                        |
| 57           | ins              | 1            |                                | 626                |                    | 18                                     |
| 58*          | ins              | 1            |                                | 683                |                    | 1, 18, 19, 20, 21                      |
| 59           | ins              | 1            |                                | 691                |                    | 4                                      |
| 60*          | ins              | 1            |                                | 841                |                    | 1, 11, 14, 21, 25                      |
| 61           | ins              | 2            |                                | 666                |                    | 11                                     |
| 62           | ins              | 4            |                                | 704                |                    | 4                                      |
| 63*          | ins              | 6            |                                | 288                |                    | 2, 12, 24                              |
| 64           | ins              | 7            |                                | 233                |                    | 11                                     |
| 65           | repeat           | 4            | AAAA                           | 149                | 145                | 18                                     |
| 66           | repeat           | 4 (8)        | AATC[ AATC]                    | 337                | 345                | 18 (3)                                 |
| 67           | repeat           | 5            | AATAC                          | 278                | 284                | 4                                      |
| 68*          | repeat           | 5            | TTGAA                          | 327                | 322                | 2, 3, 18                               |
| 69           | repeat           | 6            | TTCACC                         | 389                | 396                | 12                                     |
| 70*          | repeat?          | 6            | C(A/G)TT<br>(C/T)(A/T)         | 606                | 612                | 3, 5, 8, 9, 17, 18, 19, 22             |
| 71           | repeat           | 6            | AACTTA                         | 782                | 776                | 9                                      |
| 72           | repeat           | 7            | GATCAAA                        | 360                | 380                | 1                                      |
| 73*          | repeat?          | 7            | (C/T)TA(C/T)-<br>(T/A)C(T/G/A) | 619                | 612                | All (except 5, 9)                      |
| 74*          | repeat?          | 7            | GT(GCA)A(CT)-<br>A(CT)         | 673                | 682                | All (except 5, 6, 7, 8, 10,<br>12, 13) |
| 75           | repeat           | 20           | GATCAAAATCA-<br>TTCACTCCAT     | 360                | 380                | 6                                      |

of the 26 taxa. Invariant sites and autapomorphic base changes were removed from the analysis using the "Ignore Uninformative Characters" option. *trnL5'/F* sequences for all taxa are available from GenBank (see Table 1 for accession numbers) or can be obtained from the authors upon request. Insertion/deletion events (indels) were scored as binary characters (Table 2), following the recommendations of Wojciechowski et al. (1993), with gaps treated as missing. Primary sequence lengths and G/C contents were determined in Amplify 1.2 (Engels, 1993). These values were manually recalculated for those sequences with ambiguous nucleotide characters (e.g., N, Y, R), which are unacceptable to the program.

Sequence data were analyzed using PAUP version 3.1.1 (Swofford, 1993). Phylogenetic reconstruction was performed on unweighted characters by heuristic searches with "simple," "closest," and "furthest" addition of taxa. Heuristic searches employing a random-addition sequence of 1000 rep-

licates were also conducted to search for other islands of most parsimonious trees (Maddison, 1991).

Three separate data sets were analyzed. The first excluded all potentially phylogenetically informative indels, and the second included all indels. The third data set included only those potentially phylogenetically informative indels greater than 2 bp in length. This follows the recommendations of van Ham et al. (1994) and Lloyd and Calder (1991), who suggested that most of the homoplasy in insertion/deletion events is accounted for by smaller indels. Strict and 50% majority rule consensus trees (Margush & McMorris, 1981) were constructed for the set of equally most-parsimonious cladograms. The distribution of phylogenetically informative characters (point mutations and indels) on tree topologies was examined using MacClade version 3.0 (Maddison & Maddison, 1992).

Bootstrap (Felsenstein, 1985) and decay (Bremer, 1988; Donoghue et al., 1992) analyses were used to estimate the robustness of clades. Bootstrap

Table 3. Sequence characteristics of the *trnL* intron, *trnL*/F spacer, and combined *trnL*–*trnL*/F non-coding region sequenced in this study.

|  | <i>trnL</i> intron | <i>trnL</i> /F spacer | Combined<br>( <i>trnL</i> intron + <i>trnL</i> /F<br>spacer) |
|--|--------------------|-----------------------|--|
| Length range (bp)                                | 424–453            | (255)308–345          | (685)733–793   |
| Length mean (bp)                                 | 437.50             | 329.54                | 767.65   |
| Aligned length (bp)                              | 505                | 369                   | 874  |
| G + C content range (%)                          | 33.6–36.2          | 33.8–38.1             | 33.4–36.3  |
| G + C content mean                               | 34.9               | 35.5                  | 35.1   |
| Sequence divergence (%)                          | 1.1–6.4            | 1.2–11.7              | 1.0–7.7  |
| Number of variable sites                         | 96 (19.0%)         | 123 (33.3%)           | 219  |
| Number of potentially informative sites          | 43 (8.5%)          | 58 (15.7%)            | 101  |
| Number of constant sites                         | 409 (81.0%)        | 246 (66.7%)           | 655  |
| Number of autapomorphic sites                    | 53 (10.5%)         | 65 (12.9%)            | 118  |
| Number of indels                                 | 31                 | 44                    | 75   |
| Indel size range (bp)                            | 1–29               | 1–20 (82)             | 1–82   |
| Ratio of indels to potentially informative sites | 1:1.39             | 1:1.32                | 1:1.57   |

analyses employed 100 replicates of heuristic (SIMPLE addition sequence) searching. Decay analyses were performed using a converse constraint (ENFORCE CONVERSE command) method (Baum et al., 1994). The amount of phylogenetic information in the parsimony analysis was assessed by use of the consistency index (C.I.; Kluge & Farris, 1969) and the retention index (R.I.; Farris, 1989).

### RESULTS

Length variation for the entire *trnL* intron ranged from a low of 424 bp in *Matricaria* to a high of 453 bp in *Gazania* (Table 3). The proportion of nucleotide differences ranged from 1.1 to 6.4% between all species of Asteraceae, and from 2.7 to 6.4% between species of the Barnadesioideae and the rest of the Asteraceae (Table 3). The *trnL* intron had an average G/C content of 34.9% (33.6 to 36.2%) (Table 3).

The complete *trnL*/F intergenic spacer (corresponding to positions 49876–50231 in the *Nicotiana* genome; Fig. 1) was sequenced for all taxa in this study, and ranged in length from 255 bp in *Tagetes* to 345 bp in *Aster* (Table 3). The great range in length is somewhat misleading, because *Tagetes* has a unique 82 bp deletion; the next shortest sequence was that of *Osteospermum* (308 bp) (Table 3). The proportion of nucleotide differences in the spacer is greater than that found in the *trnL* intron and ranges from 1.2 to 11.7% between all species of Asteraceae, and from 2.2 to 10.0% between the Barnadesioideae and the ingroup (Table 3). Like the intron, the spacer has an average G/C content of 35.5% (33.8 to 38.1%) (Table 3).

Within Asteraceae, the proportion of nucleotide differences in the combined spacer and intron sequences ranged from 1.0 to 7.7% (Table 3). Total average A/T content was 64.9%, whereas G/C content was 35.1% on average (Table 3). A total of 101 sites (11.3% of the sequence length) provided potential phylogenetic information; all other sites (87.2%) were either invariant or autapomorphic (Table 3).

Seventy-five indels (Tables 2, 3), ranging in length from 1 to 82 bp, were needed to align sequences. Deletions relative to the outgroup taxa accounted for 71% (53/75) of the indels, unique sequence insertions 14.5% (11/75), and insertions that are repeats of adjacent sequence also accounted for 14.5% of the indels (Table 2). Thirty-two of the indels (Table 2) are phylogenetically informative and support relationships based on nucleotide substitutions alone (Figs. 2–4). Many more of the 1 and 2 bp (hereafter referred to as “small”) indels (64%) were homoplasious (Table 2, Fig. 3), when compared with those 3 bp and greater (35%; hereafter called “large” indels; Table 2, Fig. 4).

### PHYLOGENETIC RECONSTRUCTIONS

All three analyses (Figs. 2–4) show similar phylogenetic relationships within Asteraceae. In the 50% majority-rule trees (Figs. 2–4), branches not appearing in the strict consensus are indicated by dotted lines. The phylogenetic analysis of the sequence data excluding all indels yielded 180 equally parsimonious trees of 234 steps (C.I. = 0.61; R.I. = 0.63; Fig. 2). The data set including all indels produced 244 trees, 293 steps in length (C.I. = 0.61; R.I. = 0.64; Fig. 3).



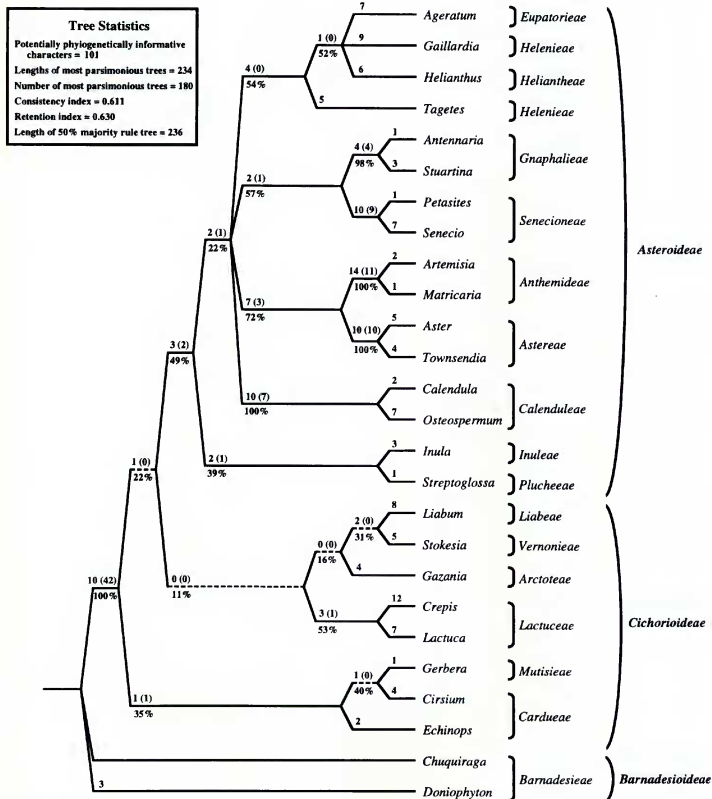


Figure 2. The 50% majority rule consensus tree of 180 equally parsimonious trees resulting from phylogenetic analysis of sequence data of the *trnL* intron and the *trnL/F* intergenic spacer using all informative base pairs, but excluding all indels. Branches that did not appear in the strict consensus tree are indicated by dashed lines. The tree gives the number of apomorphies above the branches, decay index values (in parentheses) also above the branches, and bootstrap values given as percentages below each branch. Taxon labels are from left to right: genera, tribes, and subfamilies.

whereas the data set including only large indels yielded 258 trees, 267 steps long. The latter trees have the highest consistency and retention indices of all three analyses (C.I. = 0.62; R.I. = 0.65; Fig. 4). Island searches (Maddison, 1991) on the data sets did not reveal any islands of shorter length trees.

#### TOPOLOGY OF MAJOR CLADES

All trees (Figs. 2–4) indicate that Asteroideae are monophyletic and place a clade or clades containing part of Cichorioideae, including members of tribes Liabeae, Vernonieae, Arctoteae, and Lactuceae, as sister(s) to the Asteroideae clade. Decay index values





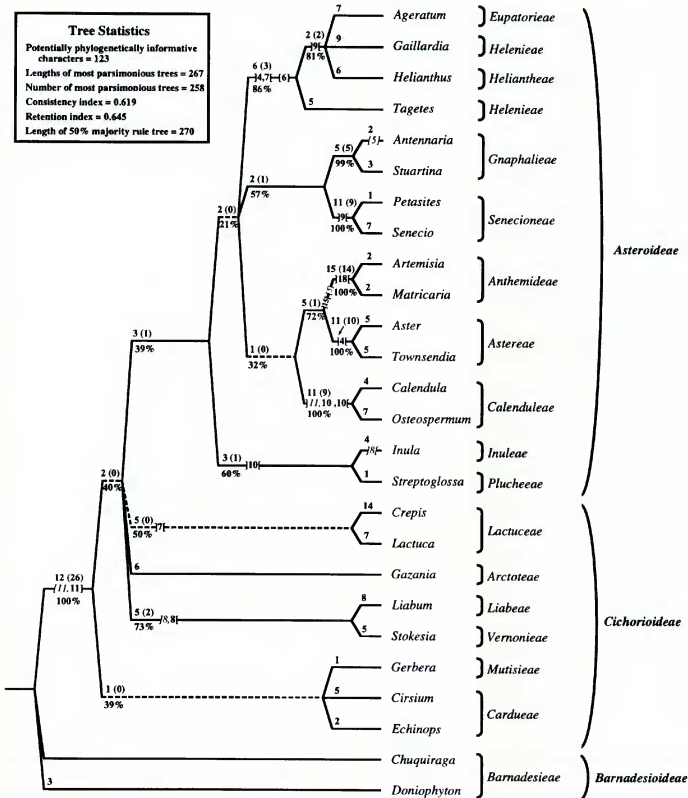


Figure 4. The 50% majority rule consensus tree of 258 equally parsimonious trees resulting from phylogenetic analysis of sequence data of the *trnL* intron and the *trnL/F* intergenic spacer using all informative base pairs, but excluding all small (1 and 2 bp) indels. Branches that did not appear in the strict consensus tree are indicated by dashed lines. The tree gives the number of apomorphies (including indels) above the branches, decay index values (in parentheses) also above the branches, and bootstrap values given as percentages below each branch. Large phylogenetically informative insertions are shown with bp length enclosed in [ ], deletions are | |. **boldface** type indels are those with C.I. of 1.00, and *italic* type are the homoplasious indels. Taxon labels are from left to right: genera, tribes, and subfamilies.

TOPOLOGY OF MINOR CLADES

Clades containing members of the tribes Eupatorieae, Helenieae, and Heliantheae (the helianthoid clade) are common to all most parsimonious

trees, but are most strongly supported (SYN = 6–9; B.V. = 86–99%) in the analyses that included indels (Figs. 3 and 4). There is low support for two additional clades within the Asteroideae, one con-

taining members of the Gnaphalieae and Senecioneae (SYN = 2; D.I. = 0–1; B.V. = 57–58%) and another containing members of the Anthemideae and Astereae (SYN = 5–7; D.I. = 0–3; B.V. = 69–72%). These clades are found in all the most parsimonious trees from the data sets containing no indels and large indels only (Figs. 2, 4). In two of the analyses (Figs. 2, 3), both of the genera in the Calenduleae are part of the main Asteroideae clade, whereas in the analysis containing only large indels (Fig. 4), they are part of a weakly supported (D.I. = 0; SYN = 1; B.V. = 32%) group that is sister to the Anthemideae–Astereae clade. In both groups of trees derived from data sets containing no indels and large indels only (Figs. 2, 4), the Inuleae–Plucheeae clade is sister to the rest of the Asteroideae, whereas in the third analysis containing all indels this clade is part of a basal polytomy of a less resolved Asteroideae (Fig. 3).

Cichorioideae are a paraphyletic group in all analyses (Figs. 2, 4). The Cardueae–Mutisieae clade mentioned above received weak support in all our trees (SYN = 1; D.I. = 0–1; B.V. = 35–43%). In all the analyses (Figs. 2–4), a clade or clades representing the tribes Liabeae, Vernonieae, Arctoteae, and Lactuceae are patristically closer to the Asteroideae clade than are Cardueae and Mutisieae. All of the trees (Figs. 2–4) show Vernonieae and Liabeae as sister taxa (SYN = 2–5; D.I. = 0–2; B.V. = 31–82%). One of the trees (all indels excluded; Fig. 2) provides weak support for a relationship in which the Arctoteae is the sister group to the Vernonieae–Liabeae clade (SYN = 0–1; B.V. = 16–42%). The Lactuceae clade has weakly supported relationships in the three trees, as sister to the Arctoteae–Liabeae–Vernonieae clade (Fig. 2; B.V. = 11%), as sister to the Asteroideae (Fig. 3; SYN = 1; B.V. = 19%), and as part of a polytomy (Fig. 4).

## DISCUSSION

This study represents one of the few to use the *trnL* intron and/or *trnL/F* intergenic spacer for phylogenetic reconstruction. The initial study of Taberlet et al. (1991) introduced PCR primers for these regions and showed that they could be amplified across a broad taxonomic range from algae to bryophytes, vascular cryptogams, gymnosperms, and angiosperms. This was followed by a phylogenetic reconstruction of some Crassulaceae genera using the *trnL/F* spacer by van Ham et al. (1994), who demonstrated the utility of the sequence to reconstruct phylogeny at the family level. Gielly and Taberlet (1996) and Gielly et al. (1996) used the

*trnL* intron to produce a phylogeny for *Gentiana* (Gentianaceae), comparing it to phylogenies for the same group based on sequences of the internal transcribed spacers (ITS) of nuclear ribosomal DNA. They concluded that ITS was more informative than the chloroplast sequence for resolving phylogenies at this level, and that the *trnL* intron sequences would probably be more useful at the intergeneric level (Gielly et al., 1996). Most recently Böhle et al. (1996) employed both of the regions used in this study, along with the *trnL/T* intergenic spacer and ITS sequences, to reconstruct the phylogeny of *Echium* (Boraginaceae) in the island groups off the northwest coast of Africa and the adjacent mainland. They obtained good resolution of the major clades (especially island versus mainland clades) within the genus, and showed the utility of combining ITS and chloroplast spacers in phylogenetic reconstruction at the generic level.

In resolving relationships in the Asteraceae, we found that the combined use of base substitutions and large indels produced trees that were better supported and less homoplasious than trees produced using only base substitutions or base substitutions and all indels. Our results agree with those of other studies (van Ham et al., 1994; Lloyd & Calder, 1991) in showing that smaller indels tend to be more homoplasious.

The averages of G/C vs. A/T content we found for the *trnL* intron and *trnL/F* spacer are nearly identical [combined average = 35.1% (Table 3) and 64.9%, respectively]; this compares favorably to the relatively narrow range in G/C content (36–39%) reported in angiosperm cpDNA (Palmer, 1991).

The topologies of our trees (Figs. 2–4) largely agree with those from other studies (Bremer, 1987; Jansen et al., 1990; Jansen et al., 1991; Karis et al., 1992; Kim et al., 1992; Karis, 1993; Kim & Jansen, 1995) of tribal relationships in the Asteraceae. Our Asteroideae, consisting of the Anthemideae, Astereae, Calenduleae, Eupatorieae, Gnaphalieae, Helenieae, Heliantheae, Inuleae, Plucheeae, and Senecioneae (Figs. 2–4), is the same monophyletic group found by Bremer (1987) based on morphology, and by Jansen et al. (1991), Kim et al. (1992), and Kim and Jansen (1995) based on molecular studies. We have also found that the Cichorioideae is paraphyletic, as reported in most other studies (Bremer, 1987; Karis et al., 1992; Kim & Jansen, 1995). The exceptions to a paraphyletic Cichorioideae are seen in the *rbcl* (Kim et al., 1992) and RFLP studies (Jansen et al., 1990; Jansen et al., 1991). The *rbcl* study, however, lacked representation from critical taxa like

the Inuleae s. str., Plucheeae, and Gnaphalieae, taxa that cause notable topological differences within the Cichorioideae when excluded in our analysis (results not shown). The *rbcL* study (Kim et al., 1992) portrayed relationships within the Cichorioideae largely incongruent with those suggested by ours and the above-mentioned studies. A recent reanalysis (Mishler et al., 1996) of the RFLP studies has found a paraphyletic Cichorioideae and has called into question the original methods of analysis (Jansen et al., 1990; Jansen et al., 1991) of those data.

The Mutisieae and Cardueae form a monophyletic group (Figs. 2–4) that is sister to a clade consisting of the remainder of the Cichorioideae and Asteroideae. Similar basal positions for the Mutisieae and Cardueae are found in morphological (Bremer, 1987; Karis et al., 1992) and most molecular-based (Jansen et al., 1990; Jansen et al., 1991; Kim & Jansen, 1995) phylogenetic reconstructions. There is some weak evidence (Fig. 2) that the Cardueae may be paraphyletic, as suggested by Ditttrich (1977). He split the Cardueae into three separate tribes, of which two, the Echinopeae and Cardueae s. str., were represented in our study (by *Echinops* and *Cirsium*, respectively).

As in many other studies (Bremer, 1987; Jansen et al., 1991; Karis et al., 1992; Kim et al., 1992), the relationships of the Lactuceae, Arctoteae, Liabeae, and Vernonieae (LALV), which form the remainder of the Cichorioideae, were largely unresolved in our investigation. We have only weak evidence for a monophyletic LALV group (Fig. 2), and Kim and Jansen (1995) also found only modest support (SYN = 3; deletion = 1) for the monophyly of this group. Most studies (Bremer, 1987; Jansen et al., 1990; Jansen et al., 1991; Kim & Jansen, 1995), including ours (Figs. 2–4), show Liabeae and Vernonieae as sisters, except for the *rbcL* study by Kim et al. (1992). Although Liabeae were once placed in Senecioneae (Robinson, 1983; Bremer, 1987), it is now clear that they are quite distinct from that tribe and are indeed most closely related to Vernonieae. It has been suggested that Vernonieae and Liabeae should be united (Jansen & Stuessy, 1980), although it appears that there are morphological synapomorphies that warrant their recognition as distinct lineages (Bremer, 1987).

We now turn our attention to the Asteroideae clade. The recent work of Anderberg (1989, 1991a, 1991b, 1991c) and Karis (1993) has shown that the Inuleae sensu Merxmüller et al. (1977) should be considered as three separate tribal lineages: the Inuleae s. str., Gnaphalieae, and Plucheeae. Although Anderberg presented strong cases for separation of

the tribes, some studies (Kim et al., 1992; Jansen et al., 1991; Bremer et al., 1992) have chosen not to address the "Inuleae problem." Our current long-term research into the molecular phylogenetics of the Gnaphalieae necessitates that we first resolve the sister-group relationships of the Gnaphalieae. We have thus included members of all three of Anderberg's tribes, and our results corroborate the morphological (Anderberg, 1989, 1991a, 1991b, 1991c; Karis, 1993) and single-molecular analysis (Kim & Jansen, 1995) in indicating that the "old" Inuleae are not a monophyletic lineage. In all of our analyses, the Inuleae s. str. and the Plucheeae are sister taxa, and these in turn are sister to the remainder of the Asteroideae in two analyses (Figs. 2, 4). Kim and Jansen (1995), using *ndhF*, showed a strong sister relationship between the Plucheeae and Inuleae, but the base of their Asteroideae was not resolved finely enough to show the sister relationships of that clade. Our topological relationships on the other hand were nearly identical to those of Karis (1993). Only the RFLP-based study of Keeley and Jansen (1991), which included members of all three tribes, showed the "old" Inuleae to be monophyletic. Therefore, based on the available evidence, the segregation of the Gnaphalieae from the "old" Inuleae seems warranted, although the circumscription of the Plucheeae is still unresolved. The sister relationships of the Gnaphalieae remain controversial. In our analysis (Figs. 2–4), the Gnaphalieae are sister to the Senecioneae. Karis (1993) showed them as sister to a clade containing the Astereae and Anthemideae, Jansen et al. (1991) as sister to the Inuleae (represented by *Inula*), Keeley and Jansen (1991) as sister to a clade consisting of the Inuleae and Plucheeae, and Kim and Jansen (1995) in an unresolved clade containing the Calenduleae, Astereae, and Anthemideae. The sister relationships of the Gnaphalieae remain unresolved due to the discordance among studies.

The sister relationships of the Astereae seem less controversial (Zhang & Bremer, 1993). We have shown them to be a well-supported sister group to the Anthemideae (Figs. 2–4), as also seen in the morphological study of Karis (1993) and the molecular studies of Jansen et al. (1991), Keeley and Jansen (1991), Kim et al. (1992), and Kim and Jansen (1995). Only Bremer (1987) portrayed them in a different relationship, as sister to the Eupatorieae. The relationships of the Calenduleae are controversial, and in only one of our analyses (Fig. 4) are their affinities to other tribes resolved, i.e., as sister to the Astereae–Anthemideae clade. Most morphological analyses do not show this relationship (Bre-

mer, 1987; Karis, 1993), while other molecular analyses (Kim et al., 1992; Kim & Jansen, 1995) support our findings. Interestingly, RFLP's in cpDNA (Jansen et al., 1991; Keeley & Jansen, 1991) show the Calenduleae as sister to the Senecioneae, which has been the traditionally recognized relationship since the time of Bentham (1873).

The helianthoid clade, including the Eupatorieae, Helenieae (Tageteae, pro parte of some authors), and Heliantheae, is a strong monophyletic group in all our analyses (Figs. 2–4). Problems arise when trying to resolve relationships and circumscribe tribes within the helianthoid clade because it appears to contain a badly understood series of phylogenetically basal branches forming successive sister groups to the rest. The combined evidence suggests that some of the tribes in the helianthoid clade are paraphyletic and need to be re-examined.

*Tagetes* was treated as part of the Helenieae by Bremer (1994), as a member of subtribe Pectidiinae (in Heliantheae) by Robinson (1981), and as the type genus of the tribe Tageteae by many authors from Cassini (1826) to Karis (1993). Our results have part of the Helenieae (*Tagetes*) as sister to a group consisting of the Eupatorieae, Helenieae (*Gaillardia*), and the Heliantheae, a disposition common to other molecular studies (Jansen et al., 1990; Jansen et al., 1991; Keeley & Jansen, 1991; Kim et al., 1992). Phylogenetic analyses using morphology (Bremer, 1987; Karis, 1993) and *ndhF* (Kim & Jansen, 1995) did not provide enough resolution to reveal relationships among most of the genera in the helianthoid clade.

The remainder of the helianthoid clade forms an unresolved polytomy containing the Heliantheae, the Eupatorieae, and the Helenieae (sensu Bremer, 1994). The Helenieae are represented by *Gaillardia*, which some authors (Robinson, 1981; Karis, 1993) have included in the Heliantheae (as the type genus of subtribe Gaillardiiinae). Our analysis does indicate that the Heliantheae in the sense of Bremer (1994), Robinson (1981), and Karis (1993), are closely related to the Eupatorieae. This is a relationship that is also reflected by a number of additional molecular analyses including those of Keeley and Jansen (1991), Jansen et al. (1991), Kim et al. (1992), and Kim and Jansen (1995). Bremer's (1987) morphological analysis showed that Astereae and Eupatorieae were sister taxa, whereas Karis (1993) portrayed a close relationship between helianthoid elements and the Eupatorieae.

In conclusion, our phylogenetic analysis of the tribes of the Asteraceae produced trees largely con-

gruent with other hypotheses based on both morphological and molecular data sets. Asteroideae are a monophyletic group, but Cichorioideae are paraphyletic. The primary clades of Cichorioideae are Mutisieae–Cardueae, Liabeae–Vernonieae; those of Asteroideae are Inuleae–Plucheeae, Astereae–Anthemideae, Senecioneae–Gnaphalieae, and the helianthoid clade (Helenieae, Heliantheae s. str., and Eupatorieae). The Inuleae–Plucheeae clade is sister to the remainder of the Asteroideae. The paraphyly of the “old” Inuleae (sensu Merxmüller et al., 1977) has been confirmed by our analysis. Calenduleae are sister to the Astereae–Anthemideae clade in some trees. A clade consisting of Lactuceae, Arctoteae, Verononieae, and Liabeae was also present in some most-parsimonious trees.

Our study illustrates the utility of the *trnL* intron and *trnL/F* intergenic spacer for resolving the relationships among tribes in the largest dicot family, Asteraceae. Using approximately 874 bp (Table 3), we were able to produce a phylogeny that shows a similar level of resolution to that produced by Kim and Jansen (1995) using 2200–2300 bp of *ndhF*. Comparison of the divergence values in the 17 taxa shared by our study and that of Kim and Jansen (1995) revealed that the combined *trnL* intron and *trnL/F* spacer evolves at a rate that is 1 to 1.28 times faster than *ndhF*. Further resolution could also be expected if additional taxa and the ca. 620–700 bp of *trnL/T* intergenic spacer were added to our analyses. Another chloroplast sequence, *rbcL*, which is 1428 to 1458 bp long in the Asteraceae and is often used in phylogeny reconstruction at the family level and above, did not provide as much resolution of the tribal relations in Asteraceae (Kim et al., 1992) as did *ndhF* (Kim & Jansen, 1995). RFLPs of chloroplast DNA, although providing fairly good resolution of relationships in the Asteraceae, resulted in several equally parsimonious trees that had moderately large amounts of homoplasy (C.I. = 0.54) (Jansen et al., 1990; Jansen et al., 1991). Additionally, that study was labor-intensive, requiring eleven restriction enzymes to produce 328 phylogenetically informative sites, and the methods of cladistic analysis of the RFLP data (Jansen et al., 1990; Jansen et al., 1991) have recently been criticized by Mishler et al. (1996). The sequences used in the present study have three advantages over the other commonly used gene regions: (1) they are easy to amplify across a wide taxonomic range because the universal primers designed by Taberlet et al. (1991) are placed in highly conserved tRNA genes; (2) the primers used to amplify the region can also be used to sequence it entirely using manual methods; and (3) the numer-

ous large indels provide additional phylogenetic information. For phylogenetic reconstruction at the family level the *trnL* intron, *trnL*/F intergenic spacer, and the *trnL*/T intergenic spacers may represent an ideal sequence, providing levels of resolution similar to those of longer gene sequences (*rbcL* and *ndhF*), but requiring much less labor to generate data.

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